



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US96/00075  <b>(22) International Filing Date:</b> 11 January 1996 (11.01.96)  <b>(30) Priority Data:</b> 08/469,387      6 June 1995 (06.06.95)      US  <b>(71) Applicant:</b> WISCONSIN ALUMNI RESEARCH FOUNDATION [US/US]; P.O. Box 7365, Madison, WI 53707-7365 (US).  <b>(72) Inventors:</b> JOHNSON, Eric, A.; 3901 Council Crest, Madison, WI 53711 (US). GOODNOUGH, Michael, C.; 6914 Harvest Hill Road, Madison, WI 53717 (US).  <b>(74) Agent:</b> KRYSHAK, Thad; Quarles & Brady, 411 East Wisconsin Avenue, Milwaukee, WI 53202-4497 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AZ, BY, KZ, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> ANALOGS OF BOTULINUM TOXIN AND PHARMACEUTICAL COMPOSITIONS OF BOTULINUM TOXIN		
<b>(57) Abstract</b>  Novel analogs of botulinum toxin have amino acid residues which are more resistant to degradation in neuromuscular tissue than the amino acid residues in the corresponding natural toxin. Pharmaceutical compositions of botulinum toxin which contain chelating agents for metallic ions and have longer half-lives in neuromuscular tissue than currently available preparations also are disclosed.		

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ANALOGS OF BOTULINUM TOXIN AND PHARMACEUTICAL  
COMPOSITIONS OF BOTULINUM TOXIN

Field of the Invention

The present invention relates to novel analogs of the botulinum toxin and pharmaceutical compositions containing botulinum toxin.

Background of the Invention

The most serious form of bacterial food poisoning is botulism which is caused by toxins produced by *Clostridium botulinum*. The toxins are usually preformed by the causative organism in foods and subsequently absorbed through the intestinal tract and transported via the circulatory system to motor nerve synapses where their action blocks normal neural transmissions. Seven known serotypes of *botulinum* toxins (type A to G) with similar toxic activity but which differ antigenically have been isolated and characterized. Serotype A toxin is the predominant cause of botulism in the United States while type B toxin is most prevalent in Europe.

A single batch of crystalline type A botulinum toxin complex was prepared in 1979 by E. J. Schantz of the Food Research Institute/Department of Food Microbiology and Toxicology at the University of Wisconsin-Madison, U.S.A. which has been used medically to treat hyperactive muscle disorders such as strabismus, blepharospasm, spasmodic torticollis, and many other diseases (Jankovic and Hallet "Therapy with Botulinum Toxin" (1994)). Treatment involves injection of nanogram quantities of the toxin complex directly into the neuromuscular tissue of hyperactive muscles. The botulinum neurotoxin in the toxin complex inhibits the release of acetylcholine across the synaptic junction causing a decrease in the activity of the injected muscles.

Type A neurotoxin produced by *C. botulinum* is present as part of a toxin complex of at least seven different noncovalently bound proteins. Both the toxin complex and the purified neurotoxin can be used in medicine and both are referred to as "botulinum toxin" herein.

High quality type A toxin complex has a specific toxicity of  $3 \times 10^7$  mouse intraperitoneal 50% lethal doses ( $LD_{50}$ ) per mg. The purified type A neurotoxin, that is the neurotoxin that has been chromatographically separated from the other proteins of the toxin complex, has a specific toxicity of  $9 \times 10^7$  to  $1 \times 10^8$   $LD_{50}$  per mg. In the medical field, a unit (U) is considered to be 1 mouse  $LD_{50}$ . Toxin titers are determined in female, white mice, 18-22g in weight according to the method of Schantz and Kautter as described in *Association of Official and Analytical Chemistry*, vol. 61, p. 96, (1978). Evidence is accumulating that different types of botulinum toxin may bind to different acceptors than type A and may have differences in their mode of action and clinical applications.

A major drawback to the use of pharmaceutical compositions containing either botulinum toxin complex or the pure neurotoxin in treatment of hyperactive muscle disorders is that the duration of action in neuromuscular tissue of botulinum toxin in the available products currently is relatively short (i.e., a few days to several months, depending on the indication being treated) and thus frequent injections are required by the patients.

It obviously would be advantageous to have botulinum toxin containing compositions which have longer half-lives in vivo in neuromuscular tissue than known botulinum toxin containing compositions. This would enable patients to be injected less often and would reduce the incidence of antibodies since immunologic tolerance to botulinum toxin is correlated with cumulative doses.

#### Brief Summary of the Invention

It is an object to disclose novel analogs of botulinum neurotoxin derived by mutagenesis that have longer half-lives than the corresponding natural neurotoxin.

It also is an object of the present invention to disclose pharmaceutical compositions containing botulinum toxin which have longer half-lives than known compositions.

We have discovered that the compositions containing novel analogs of natural botulinum neurotoxins that have more

resistant amino acids in degradable sites outside of the active toxin sites have longer half-lives in neuromuscular tissue than the corresponding natural botulinum toxins.

We have also discovered that pharmaceutical compositions containing a botulinum toxin and a  $\text{Ca}^{++}$  chelating agent prevents the degradation of the neurotoxin in neuromuscular tissue resulting in longer half-lives. Thus, the use of chelating agents or the preventing of the uptake of  $\text{Ca}^{++}$  in neuromuscular tissue will prolong the half-life of toxin activity.

10                    Description of Preferred Embodiment

The use of the botulinum toxin preparations with high specific toxicities (e.g. a properly prepared complex of about 30U/mg; or pure neurotoxins of about 90-100U/mg) is preferred because it reduces the amount of toxin required to obtain the necessary number of active U per vial as mandated by the United States Food and Drug Administration. This also reduces the amount of inactive toxin (toxoid) in each vial and thereby lessens the possibility of antibody formation after injection of the preparation into patients.

20            The preferred pharmaceutical compositions of the present invention have the following composition:

Genetically modified botulinum Type A neurotoxin complex or neurotoxin	100 U (after lyophilization)
Serum albumin	0.5-1 mg. per vial.

25            It may also be desired to include 1-5 mg of a chelating agent for  $\text{Ca}^{++}$  (e.g. 1-5 mg of EDTA and EGTA) and trehalose for shelf stability.

The Hall A strain of type A *C. botulinum* (deposited with the ATCC) is used to produce type A botulinum toxin. This strain is routinely used for production of type A botulinum toxin because of high toxin titers and the rapid onset of cell lysis (usually within 48 h). However, the terms "Type A toxin" and "Type A neurotoxin" as used herein are intended to cover the toxin and neurotoxin of all strains because they exhibit only minor variations in sequence.

For crystalline toxin production, cultures of the Hall A strain are grown statically in 10-20 liter volumes of toxin

production medium (TPM) consisting of 2.0% NZ amine or TT (Sheffield Laboratories, Norwich, NY U.S.A.), 1.0% yeast extract (Difco), and 0.5% dextrose, pH 7.37.4, for 5-7 days at 37°C.

5 Type A crystalline complex is purified by a modification of the method of Duff et al, J. Bacteriol, 73, pp. 42-47 (1957).

To prepare essentially pure type A neurotoxin, the type A toxin is then purified according to the method described in the Ph.D. thesis of M. C. Goodnough (Goodnough, M.C. 1994, "Characterization and stabilization of *Clostridium botulinum* toxin for medical use", University of Wisconsin-Madison, U.S.A., as adapted from Tse et al. 1982)

Briefly, toxin complex is recovered from DEAE-Sephadex A50 (Sigma Chemical Co., St. Louis, MO U.S.A.), pH 5.5, column and is precipitated by addition of 39 g of solid ammonium sulfate/100ml. The precipitated toxin complex is collected by centrifugation, dialyzed against 25 mM sodium phosphate, pH 7.9, and applied to a DEAE-Sephadex A50 column equilibrated with the same buffer. Toxin is separated from the non-toxic proteins of the complex and eluted from the column with a linear 0-0.5M sodium chloride gradient. Partially purified neurotoxin is recovered from the DEAE-Sephadex A50 column at pH 7.9 and dialyzed against 25 mM sodium phosphate, pH 7.0. The dialyzed toxin is applied to SP-Sephadex C50 (Sigma Chemical Co.) in 25 mM sodium phosphate, pH 7.0. Contaminating material does not bind to the column under these conditions. The neurotoxin is eluted with a linear 0-0.25 M sodium chloride gradient. The neurotoxin can be further purified by metal affinity chromatography, gel filtration, high pressure liquid chromatography or other methods of protein chromatography.

30 When the approach using chelating is used to prevent degradation in the neuromuscular preparations, the Ca<sup>++</sup> chelating agents preferred for use in the compositions of the present invention are EDTA and EGTA. The amount of chelating agent to be included is about 1 mg to about 5 mg for each 100U of the botulinum neurotoxin that is present either alone or calculated to be present in the botulinum toxin complex.

The addition of trehalose, allows for the recovery of active type A neurotoxin following lyophilization and storage at

ambient temperatures (37°C) which are in excess of those required for storage of the commercially available type A toxin complex (-10°C). In general, the amount of trehalose that should be present is the amount that is necessary to stabilize the composition so that it can be stored at ambient temperatures. Usually, the composition will contain about 10 mg of trehalose per vial of (50 to 100 mg/ml prelyophilized solution).

The preferred compositions will also contain an effective amount of a stabilizing protein, such as human serum albumin. Usually, the serum albumin will be present in about 0.5 mg per vial (1/10 mg/ml of prelyophilized solution). In addition to human serum albumin, other known stabilizing proteins including bovine serum albumin, can be used in the compositions of the present invention.

The preferred analogs of the botulinum neurotoxin of the present invention are those which possess the beneficial medicinal activity of the natural botulinum neurotoxin serotype and which differ from the natural botulinum neurotoxin only in that they have been genetically modified to resist proteolytic degradation in neuromuscular tissue (e.g. a threonine residue at the 540 amino acid position in place of the tryosine residue of the natural product). The genetically modified product is more resistant to proteolytic breakdown. Other residues in sensitive sites can also be modified by analogous site specific mutagenesis.

The amino acid sequence of natural Type A botulinum neurotoxin is conserved and has been determined by several laboratories. (Thompson et al. *Eur. J. Biochem*, 89, pp. 73-81 (1990). Only a few residues in non-conserved regions have been found to differ in the toxin sequences of the neurotoxins of other serotypes (Binz et al, *J. Biol. Chem.* 265, pp. 9153-9158 (1990).

It has now been discovered that analogs of botulinum neurotoxin, which differ from the natural neurotoxin in that they contain modification of susceptible amino acid pairs (e.g. tyrosine, arginine, tryptophan pairings) in the sites subject to degradation in neuromuscular tissue, are more resistant to

proteolytic degradation. The susceptible amino acids are replaced with residues that resist proteolytic degradation (e.g. tyrosine is replaced by a threonine). These modified toxins possess improved half-lives in neuromuscular tissue as compared to natural botulinum toxin.

The method for preparing the proteolytic degradation resistant botulinum neurotoxin analogs basically comprises replacing an amino acid residue in a pairing at a site(s) subject to degradation outside the active sites in the natural neurotoxin with another amino acid residue which is more resistant to acid and/or proteolytic attack or degradation.

The method of the present invention may be practiced by using cloning, site-specific mutagenesis and/or cassette mutagenesis to modify the gene encoding the natural or wild type neurotoxin and by subsequent introduction of the modified gene into a microorganism that overproduces the modified neurotoxin.

In the preferred method the analogs of the neurotoxin are prepared by the use of site-directed mutagenesis.

An especially preferred method comprises preparing a DNA transfer vector containing a DNA sequence which encodes for the neurotoxin but which has an amino acid residue which is more resistant to degradation in a site which is subject to degradation *in vivo* in the natural neurotoxin; transforming the DNA transfer vector into a microorganism, such as E. coli; and then culturing the microorganism under conditions suitable for the expression of the botulinum neurotoxin analog.

Representative of the analogs of Type A botulinum neurotoxin that can be made in the light chain (the catalytic region of botulinum neurotoxin) are the following:

(a) An analog having a threonine residue in place of the tyrosine residue at site 540.

(b) An analog having an asparagine residue in place of the arginine residue at site 1183.

The pharmaceutical compositions of the present invention are preferably supplied as lyophilized products. For lyophilization, a solution containing the active crystalline toxin, neurotoxin or genetically modified toxin or analog, a protective protein, such as serum albumin and the water for



injection (USP) is placed in glass vials, Teflon lined screw cap closures are fastened loosely, and the solutions quickly frozen in liquid nitrogen. Additionally, the formulations may include a  $\text{Ca}^{++}$  chelating agent and a carbohydrate, such a trehalose for shelf stability. The frozen solutions in the vials are placed into a lyophilization vessel which is then immersed in liquid nitrogen. The vessel is then connected to a freeze-drier. When the pressure drops below ca. 60 mTorr, the liquid nitrogen jacket is removed. Pressure is maintained at or below 30-60 mTorr and condenser temperature constant at  $-60^{\circ}\text{C}$ . The vials and their contents are allowed to come to room temperature and drying continued at ambient temperature over the next 18-24 h. At that time the vials are tightly capped. The lyophilized preparations were usually reconstituted by the addition of 1.0 ml of distilled water or 0.85% saline for injection.

The preferred compositions also differ from the commercially available products in that they do not contain sodium chloride which has been found to reduce the recovery of active toxin following lyophilization.

The primary advantage of preferred compositions of the present invention is their longer half-lives in the presence of tissue proteolytic enzymes.

The invention is further illustrated by the following examples.

#### Example 1

##### Site-directed mutagenesis

A suitable plasmid is digested with restriction of enzymes, such as EcoRI and BamHI. A DNA fragment, containing the entire type A neurotoxin gene and its promoter region is isolated and subcloned into the EcoRI/BamHI sites of the plasmid. The oligonucleotide primers, obtained from Genetic Designs, Inc., (Houston, TX U.S.A.), are designed to be complementary to the single-strand template DNA and to contain appropriate mismatches. Synthesis of genes for the analogs and selection are performed using a kit of Oligonucleotide-directed Mutagenesis System, version 2 (Amersham). Nucleotide sequences of the mutant genes obtained can be confirmed by the dideoxy sequencing method. The enzyme (EcoRI/BamHI) fragments,

containing the mutant genes are subcloned from each recombinant into the EcoRI and BamHI sites of vectors, and introduced into a suitable E. coli strain, such as HB101 (ATCC 33694).

The E. coli cells, carrying appropriate recombinant  
5 plasmids that specify the mutant analogs are cultivated overnight in LB media, containing 100 µg/ml Ampicillin, at 37°C with vigorous shaking. Cells are harvested by centrifugation at 5,000 x g, washed and suspended in 50 mM MPOS buffer (pH 7.0) containing 10 mM MgSO<sub>4</sub> and 1 mM CoCl<sub>2</sub>. For preparation of cell  
10 extracts, cells are broken by two passages through a French pressure cell at 18,000 p.s.i., and the debris removed by centrifugation at 12,000 x g for 20 min. The cell extracts are stirred at 85°C for 20 min. and centrifuged at 12,000 x g for 30 min. The soluble fractions from the cell extracts are loaded  
15 onto a DEAE-Sepharose CL-6B columns (4.0 cm x 32 cm), pre-equilibrated with 50 mM MOPS buffer pH 7.0 and proteins are eluted with linear NaCl salt gradient (0.0-0.5M) in the same buffer. Fractions containing significant Botulinum toxin activity are pooled. The mutant analog proteins can be  
20 identified by SDS-PAGE analysis from the fractions.

#### Example 2

The light chain of botulinum toxin (the chain that causes poisoning) was purified and incubated with brain extract of rabbits. Using conventional techniques it was shown that the  
25 toxin was cleaved by proteases present in the nerve extract. The cleavage was found to be dependent on calcium. Using a purified Ca<sup>++</sup>-dependent protease it was shown that the light chain was cleaved at only one site (540) yielding two fragments of about the same size. The cleavage was Ca<sup>++</sup> dependent and it  
30 was found that it could be prevented by using chelators that bind CA, including EGTA and EDTA.

It will be apparent to those skilled in the art that a number of modifications and changes can be made without departing from the spirit and scope of the present invention.  
35 Therefore, it is intended that the invention be limited only by the claims.

We claim:

1. An analog of botulinum toxin, said analog differing from the corresponding natural botulinum toxin in that the analog has a more stable amino acid residue in a pairing at a degradable site in the natural toxin than the amino acid residue  
5 of the natural toxin.
2. An analog of claim 1 in which the botulinum toxin is Type A botulinum neurotoxin.
3. An analog of claim 1 in which the analog has a threonine residue in place of a tryosine residue in the degradable site.
4. An analog of claim 1 in which the analog has an asparagine residue in place of an arginine residue in the degradable site.
5. A method of prolonging the half-life of a botulinum toxin in neuromuscular tissue which comprises administering into neuromuscular tissue with the botulinum toxin a safe and effective amount of a chelating agent that binds the metallic  
5 ions required by an enzyme that is present in the tissue and thereby inhibits said enzyme's ability to degrade the botulinum toxin and adversely affect its half-life.
6. A pharmaceutical composition comprising:
  - (a) a member selected from the group consisting of botulinum toxin complex, botulinum neurotoxin and analogs of the neurotoxin which possess the desired botulinum toxin activity;  
5 and
  - (b) a safe amount of a chelating agent which is effective to bind metallic ions and thus prevent enzymes from adversely affecting the half-life of the member in vivo in neuromuscular tissue.
7. A composition of claim 6 which contains a stabilizing protein.

8. A composition of claim 6 which contains trehalose.

9. A composition of claim 6 in which the chelating agent is selected from EDTA and EGTA.

10. A lyophilized pharmaceutical composition having botulinum toxin biological activity, said composition comprising a member selected from the group consisting of botulinum toxin complex, botulinum neurotoxin and analogs of botulinum neurotoxin which possess the beneficial medicinal activity of the neurotoxin and an effective amount of a chelating agent that prevents an enzyme in neuromuscular tissue from degrading of said member.

11. A composition of claim 10 which contains a chelating agent selected from the group consisting of EDTA and EGTA.

# INTERNATIONAL SEARCH REPORT

International Application No

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A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A61K38/16 C07K14/33 //A61K31/225

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,95 05842 (THE JOHNS HOPKINS UNIVERSITY) 2 March 1995 see the whole document ---	1-11
A	WO,A,94 00481 (ASSOCIATED SYNAPSE BIOLOGICS) 6 January 1994 see the whole document ---	1-11
A	EP,A,0 593 176 (WISCONSIN ALUMNI RESEARCH FOUNDATION) 20 April 1994 see the whole document ---	1-11
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Further documents are listed in the continuation of box C.



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International Application No

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	205TH ACS (AMERICAN CHEMICAL SOCIETY) NATIONAL MEETING, DENVER, COLORADO, USA, MARCH 28-APRIL 2, 1993. ABSTR. PAP. CHEM. SOC. 205 (1-2). 1993, ABSTR. NO. 149, XP002002967 GOODNOUGH, M.C. AND E.A. JOHNSON: "Stabilization of Clostridium-Botulinum Neurotoxin during Lyophilization" ---	1-11
A	MICROBIOLOGICAL REVIEWS, vol. 56, 1992, pages 80-99, XP002002968 SCHANTZ, E.J. AND E.A. JOHNSON: see page 81 - page 89 ---	1-11
A	JANKOVIC, J. AND M. HALLETT (ED.). NEUROLOGICAL DISEASE AND THERAPY, VOL. 25. THERAPY WITH BOTULIMUN TOXIN. XXVI+608P. MARCEL DEKKER, INC.: NEW YORK, NEW YORK, USA; BASEL, SWITZERLAND, 1994, pages 41-49, XP002002969 SCHANTZ, E.J. AND E.A. JOHNSON: see page 46 - page 47 -----	1-11

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Information on patent family members

International Application No

PCT/US 96/00075

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9505842	02-03-95	US-A- 5437291	01-08-95
WO-A-9400481	06-01-94	AU-B- 4646393	24-01-94
		CA-A- 2138020	06-01-94
		EP-A- 0654040	24-05-95
EP-A-0593176	20-04-94	JP-A- 6192118	12-07-94